

*Application  
for  
United States Letters Patent*

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109027-99ET260

**To all whom it may concern:**

Be it known that **Joshua S. Salafsky and Kenneth B. Eisenthal**

have invented certain new and useful improvements in

**ATTACHMENT OF SECOND HARMONIC-ACTIVE MOIETY TO MOLECULES FOR DETECTION OF  
MOLECULES AT INTERFACES**

of which the following is a full, clear and exact description.

**ATTACHMENT OF SECOND HARMONIC-ACTIVE MOIETY TO  
MOLECULES FOR DETECTION OF MOLECULES AT INTERFACES**

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The invention disclosed herein was made with Government support under grant number CHE-96-12685 from the National Science Foundation and grant numbers DE-FG02-91ER and DE-FG02-14226 from the Department of Energy. Accordingly, the U.S. Government has certain rights in this invention.

**Background Of The Invention**

Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Second harmonic generation (SHG) is a powerful spectroscopic tool for studying interfacial regions at the molecular scale, but to date has been confined mainly to non-biological systems. Recently, however, SHG has been extended to the study of a SH-active moiety at a supported lipid membrane system (Salafsky and Eienthal, 2000a), a useful model for biological studies, and to the detection of protein adsorption at charged interfaces through the indirect effect the protein has on polarized water molecules near the

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side-chains. Unlike detection with fluorescent labels, SHG-labels have the important advantage that

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labels should find use in a variety of biological applications including studies of protein-protein, protein-membrane, and cell-cell interactions. SHG-

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labels can also be used to study other systems such as nanoparticle surfaces and polymer systems (polymer beads). In turn the labelled nanoparticle or labelled polymer bead can be used for example as a sensor of molecules in the surrounding medium.

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Summary Of The Invention

5 The present invention provides a method for detecting a molecule at an interface, which comprises labeling the molecule with a second harmonic-active moiety and detecting the labeled molecule at the interface using a surface selective technique.

10 The invention also provides a method for detecting a molecule in a medium, which comprises:

- (a) labeling a surface with a second harmonic-active moiety wherein the second harmonic-active moiety specifically interacts with the molecule to be detected,
- 15 (b) exposing the surface to the medium thereby creating an interface at the surface,
- (c) detecting the second harmonic-active moiety at the interface by measuring a signal generated using a surface selective technique, and
- 20 (d) detecting a change in the signal when the molecule interacts with the second harmonic-active moiety, thereby detecting the molecule in the medium.

25 This invention provides a method for determining the orientation of a molecular species within a planar surface, which comprises:

- (a) labeling the species with a second harmonic-active moiety which specifically binds to the species;
- 30 (b) determining the orientation of the second harmonic-active moiety with respect to the species;

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- (c) measuring the polarization of second-harmonic light to determine the orientation of the second harmonic-active moiety with respect to the planar surface; and
- 5 (d) determining the orientation of the species within the planar surface from the orientation of the moiety with respect to the surface as determined in step (c) and from the orientation of the moiety with respect to the species as  
10 determined in step (b).

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## Brief Description Of The Figures

Figure 1. Absorption spectra of the SH-active moiety (dye)(oxazole derivative), cytochrome (cyt) c and dye-cyt c (amine) conjugate. The SH-active moiety is covalently attached to amine groups on the protein's surface and, from the absorbance and known extinction coefficients, is bound at a mole ratio of 1.5:1 (dye:protein) in the conjugate.

Figure 2. The SH intensity spectrum of the cyt c - amine conjugate (1.5:1 dye:protein mole ratio; solid line) and cyt c -cysteine conjugate (0.4:1 dye:protein mole ratio; ◆◆◆ line) at the air-water interface at bulk concentrations of about 15  $\mu$ M. The fundamental wavelength was set to 804 nm and the spectrum displays the characteristic  $2\omega$  peak. The tail of the two-photon fluorescence is visible in the spectrum of the amine conjugate.  $I_{SH}$  (cps) is the intensity of SH light in counts per second.

Figure 3. Adsorption isotherm of the cysteine-cyt c conjugate (SH intensity vs. bulk [conjugate]) with error bars. The curve was fitted to a Langmuir adsorption model and, from this, a free energy of adsorption of  $\Delta G = -11$  kcal/mole and the number of adsorbates at the interface were determined.

Figure 4. SH intensity spectrum of the oxazole dye alone at the air-water interface. The bulk dye concentration is about 700  $\mu$ M. Although the peak SH intensity is comparable to that for the cysteine

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Detailed Description Of The Invention

The following definitions are presented as an aid in understanding this invention.

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As used herein **second harmonic** refers to a frequency of light that is twice the frequency of a fundamental beam of light. A **second harmonic-active moiety** is a substance which when irradiated with a fundamental beam of light generates a second harmonic of the fundamental.

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Having due regard to the preceding definitions, the present invention concerns a method for detecting a molecule at an interface, which comprises labeling the molecule with a second harmonic-active moiety and detecting the labeled molecule at the interface using a surface selective technique.

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In different embodiments of the invention, the surface selective technique is second harmonic generation or sum-frequency generation. Sum frequency generation (SFG) is a nonlinear, optical technique whereby light at one frequency ( $\omega_1$ ) is mixed with light at another frequency ( $\omega_2$ ) to yield a response at the sum frequency ( $\omega_1 + \omega_2$ ) (Shen, 1984, 1989). SFG is particularly useful for the detection of molecules at surfaces through their characteristic vibrational transitions and, in this case, is essentially a surface-selective infrared spectroscopy with  $\omega_1$  and  $\omega_2$  at visible and infrared frequencies.

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In different embodiments, the interface is at a membrane, a liposome, a cell surface, a viral surface, a bacterial surface, or a biosensor. In  
5 different embodiments, the interface is a vapor-liquid interface, a liquid-liquid interface, a liquid-solid, or a solid-solid interface. In one embodiment, the vapor-liquid interface is an air-water interface. In one embodiment, the liquid-  
10 liquid interface is an oil-water interface. In different embodiments, the liquid-solid interface is a water-glass interface or a benzene-SiO<sub>2</sub> interface.

The present invention provides for the use of any of  
15 the methods described herein to detect binding of a protein to a receptor on a membrane. The invention also provides for the use of any of the methods described herein to detect binding of a virus to a cell. The invention further provides for the use of  
20 any of the methods described herein to study protein-protein interaction at an interface or to study cell-cell interaction.

The invention provides a method for detecting a  
25 molecule in a medium, which comprises:

- (a) labeling a surface with a second harmonic-active moiety wherein the second harmonic-active moiety specifically interacts with the molecule to be detected,
- 30 (b) exposing the surface to the medium thereby creating an interface at the surface,

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- (c) detecting the second harmonic-active moiety at the interface by measuring a signal generated using a surface selective technique, and
- (d) detecting a change in the signal when the molecule interacts with the second harmonic-active moiety, thereby detecting the molecule in the medium.

In different embodiments of the method, the surface is on a nanoparticle or a polymer bead. In different embodiments, the surface selective technique is second harmonic generation or sum-frequency generation.

In different embodiments, the molecule to be detected is a pollutant or a charged species. In different embodiments, the pollutant is lead or polychlorinated biphenyl. In one embodiment, the charged species is a chloride ion.

In one embodiment, the interaction between the second harmonic-active moiety and the molecule to be detected is an antibody-antigen interaction.

In different embodiments, the medium contains an amount of the molecule to be detected, the change in the signal when the molecule interacts with the second harmonic-active moiety is a quantitative change, and the amount of the molecule in the medium can be determined from the change in the signal.

This invention provides a method for determining the orientation of a molecular species within a planar surface, which comprises:

- 5 (a) labeling the species with a second harmonic-active moiety which specifically binds to the species;
- (b) determining the orientation of the second harmonic-active moiety with respect to the species;
- 10 (c) measuring the polarization of second-harmonic light to determine the orientation of the second harmonic-active moiety with respect to the planar surface; and
- 15 (d) determining the orientation of the species within the planar surface from the orientation of the moiety with respect to the surface as determined in step (c) and from the orientation of the moiety with respect to the species as determined in step (b).

20 In one embodiment of the method, the orientation of the second harmonic-active moiety with respect to the species is determined using x-ray crystallography. In different embodiments, the planar surface is selected  
25 from the group consisting of an organic material surface, an inorganic material surface, a polymeric material surface, a mineral surface, a clay surface, a biological membrane surface, and a synthetic membrane surface. In different embodiments, the  
30 molecular species is selected from the group consisting of an organic species, an inorganic species, a polymeric species, a protein, a lipid, a nucleic acid, and a carbohydrate.

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This invention will be better understood from the  
Experimental Details which follow. However, one  
skilled in the art will readily appreciate that the  
5 specific methods and results discussed are merely  
illustrative of the invention as described more fully  
in the claims which follow thereafter.

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## Experimental Details

The attachment of a second harmonic-active moiety to a molecule for detection of the molecule at an interface can be illustrated by studying SHG-labeled cytochrome c at the air-water interface. Since the x-ray crystal structure of cytochrome c has been solved to atomic resolution, experiments can be designed to randomly or selectively label various amino acid side-chains on the protein's surface. For instance, because only one surface cysteine exists on cytochrome c, SHG can be used to detect the orientation of the cysteine conjugate at the air-water interface through a null angle technique. The free energy of adsorption of the SH-active moiety - protein conjugate to the air-water interface can also be measured.

## Theoretical Considerations

The production of second harmonic light from an interface can be described by the following equation:

$$I(2\omega) = \frac{32\pi^3\omega^2 \sec^2 \Theta}{c^3 \epsilon(\omega) \epsilon^{1/2}(2\omega)} \left| \bar{e}(2\omega) \cdot \chi^{(2)} : \bar{e}(\omega) \bar{e}(\omega) \right|^2 I^2(\omega) \quad (1)$$

where  $I(2\omega)$  and  $I(\omega)$  are the intensity of the second harmonic and fundamental light, respectively,  $\chi^{(2)}$  is the second-order nonlinear susceptibility tensor,  $\bar{e}(\omega)$  and  $\bar{e}(2\omega)$  the products of the Fresnel factors and the polarization vectors for the light beams,  $c$  is the speed of light,  $\epsilon$  is the index of refraction, and

$\Theta$  is the angle between the reflected harmonic light and the surface plane (Heinz, 1991). The surface nonlinear susceptibility  $\chi^{(2)}$ , neglecting local-field effects, is

$$\chi^{(2)} = N_s \langle \alpha^{(2)} \rangle \quad (2)$$

where  $N_s$  is the total number of molecules per unit area at the interface and  $\langle \alpha^{(2)} \rangle$  is the average over the orientational distribution of the nonlinear polarizabilities in these molecules. Equation (2) can be more explicitly expressed as

$$\chi_{ijk}^{(2)} = N_s \langle T_{i\lambda} T_{j\mu} T_{k\nu} \rangle \alpha_{\lambda\mu\nu}^{(2)} \quad (3)$$

following Reider and Heinz (1995), where  $\alpha_{i'j'k'}^{(2)}$  refers to the molecular nonlinear polarizability in the coordinate system of the molecule,  $T_{i\lambda}$  is the transformation tensor which relates the laboratory and molecular frames of reference, and the average is taken over the orientational distribution of the molecules at the interface. From equations 1 and 2, the intensity of second harmonic radiation is quadratic with the surface density  $N_s$  of aligned molecules.

In the use of SHG-labels for molecules (nonlinear polarizability  $\alpha_L^{(2)}$  per label), the distribution of the labels will determine the orientational average of the labels' polarizability and therefore the SH intensity. Typically, the labels will not be

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oriented in the same direction, and so the SH intensity will be reduced; in the limit of many randomly distributed labels, the SH intensity will approach zero. Labeling ratios can therefore be adjusted according to the particular molecule under study to maximize the net SH signal from several labels. One could also use 'super-labels', in which a number of individual labels are bound together in a fixed and determinate orientation with respect to each other, in order to maximize the overall hyperpolarizability. The molecules, and therefore the labels, must also exhibit a net orientation at the interface to produce a nonlinear effect. This requirement could be an advantage, however, in detecting specific interactions, for example with protein-receptor recognition which leads to a net protein orientation. Furthermore, through mutagenesis, one can specifically engineer proteins for the purpose of placing SHG-labels in pre-determined positions.

#### Methods

Amine- and sulfhydryl-specific dyes (Molecular Probes; Eugene, OR), derivatives of an oxazole dye which has been shown to be highly SH-active, were covalently attached to cytochrome c via either a surface lysine or cysteine amino acid, respectively. Cytochrome c (Horse heart, Sigma) is a soluble, globular protein of about 12 kDa molecular weight which participates in biological electron transfer reactions through its heme cofactor. The protein has, from an examination of the crystal structure

(code 1HRC - Protein Databank; 1.9 Å resolution), 19 surface-exposed lysine side-chain amino groups, the target of the acylating dye, and a single, surface sulfhydryl group (cysteine 17). The protein has a net charge of +9 at pH 7 and a maximum ground state dipole moment of 60 Debye calculated by using a standard program and the Charm charge set (Gunner et al., 1996 and references therein). Cytochrome c was covalently labeled with either an amine- or sulfhydryl-selective derivative of oxazole pyridinium, both of which carry a positive +1 charge, and purified by extensive dialysis according to prescribed procedure and previous work (Salafsky et al., 1996). The sulfhydryl-specific dye was 1-(2,3-epoxypropyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium trifluoromethanesulfonate (PyMPO epoxide), and the amine-specific dye was 1-(3-(succinimidylloxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium bromide (PyMPO, SE). Non-covalently bound dye could be completely removed by successive dialysis steps as established by a control experiment with a non-reactive oxazole dye. The degree of labeling could be determined by measuring the absorption spectrum of the protein-dye conjugate. Dye derivatives were obtained from Molecular Probes, and the parent compound has been shown to be highly SH-active at neutral pH (Salafsky and Eisinger, 2000), the latter being of importance for biological studies. Protein was reacted with the dye at a 5:1 mole ratio (dye:protein) in distilled water (sulfhydryl-specific reaction) or sodium bicarbonate at pH 8.3 (amine-specific).

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The second harmonic generation set-up has been described previously in detail (Eisenthal, 1996; and references therein). Briefly, the beam of an argon ion laser (10.5 W) is directed into the cavity of a titanium sapphire mode-locked laser (Tsunami). The output, at a repetition rate of 82 MHz with ~150 fs pulse duration (800-834 nm adjustable), was used as the fundamental light. The fundamental light was directed on to the reflecting plane of the water surface (double distilled water in a Teflon dish) at an angle of 70° to the surface normal, and was removed after the sample by a filter. Although the two-photon fluorescence of the dye is easily visible as a greenish-yellow glow, it is spectrally well-separated from the  $2\omega$  wavelength due to a Stokes shift of ~130 nm. In addition, the reflection set-up collects only a small portion of the light emitted, which further reduces the amount of detected fluorescence. The second harmonic light was collected and focused into a monochromator. For all experiments, the polarization of the fundamental was set to 45° from the normal to the laser table. Detection was accomplished using a photomultiplier tube in single photon-counting mode.

#### Results and Interpretation

The intensity of SH light generated from the air-water interface at the  $2\omega$  peak was measured to be  $50 \pm 12$  counts per second. Cytochrome c has been shown previously to adsorb to the air-water interface (Kozarac et al., 1987). When cytochrome c (without dye label) was added to the water phase, even at a 5-

10 mM concentration, the SH intensity from the interface either remained unchanged or diminished slightly, demonstrating that the heme cofactor does not contribute significantly to the SH signal through a  $\chi^{(2)}$  process, as expected since the heme macrocycle is highly centrosymmetric. This finding is also consistent with previous work in which the fundamental wavelength was tuned across the heme absorption without effect on the SH signal.

A typical absorption spectrum of the dye-protein conjugate is shown in Figure 1, along with spectra of the protein and dye separately. In Figure 1, cytochrome (cyt) c is conjugated to an amine-reactive oxazole derivative and the degree of labeling is about 1.5:1 mole (dye:cyt) using the known extinction coefficients of the protein's heme group and the dye ( $9.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  at 412 nm and  $2.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  at 415 nm, respectively). At a concentration of 15  $\mu\text{M}$  in the subphase, the amine-labeled protein at the air-water interface produces a  $2\omega$  signal of order  $10^4$  cps (Figure 2). The tail of the dye fluorescence is visible at about 480 nm and considerably smaller in magnitude than the SH peak. The SH signal of the cysteine-labeled cytochrome c was also measured (Figure 2) and exhibits the same  $2\omega$  peak as that of the amine conjugate, although the signal is smaller, around  $10^3$  cps; this is expected given the smaller dye:protein labeling ratio (0.4:1 indicating about one dye every other protein) as measured from the conjugate's absorption spectrum.

The orientation of the dye molecule in the cysteine conjugate was measured using a null angle technique (Salafsky and Eisenthal, 2000; Hicks, 1986). The SH light produced by this conjugate could be completely nulled by setting the analyzer at an angle of 30° from the surface normal, corresponding to a dye molecular orientation of 52° degrees from the normal under the assumption of a single hyperpolarizability element and a delta function in dye distribution. Because the dye orientation is measured with respect to the interfacial plane, an assumption of the dye's orientation on the surface of the protein must be made to determine the protein's orientation in the lab frame. If we assume that the dye is oriented so that its principal bond axis (and likely SH-active axis) lies normal to the protein's surface, from an examination of the x-ray crystal structure the cytochrome c is oriented so that the heme plane is about 30° degrees from the normal to the air-water interface, since the cysteine sulfhydryl group lies nearly in the plane of the heme. An adsorption isotherm of the cysteine conjugate was also measured at the air-water interface by adding small aliquots of the conjugate to the water subphase (Figure 3). By fitting the data to a Langmuir isotherm (Eisenthal, 1996), the free energy of the conjugate's adsorption to the air-water interface could be determined to be  $\Delta G = -11$  kcal/mole which indicates a strong surface activity. However, when the oxazole dye alone (+1 charge) was added to the water phase at the concentrations used for the conjugate (1-15  $\mu$ M), it produced no detectable change in the background SH signal. Only at much higher concentrations of the

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dye ( $> 0.5$  mM) was the SH signal detectable, with an intensity of several thousand counts per second (Figure 4). The free energy of the cytochrome adsorption to the air-water interface is therefore significantly larger than that for the free oxazole dye.

These results suggest the use of SHG-labels in other experiments. For instance, by designing an appropriate molecular platform at an interface - a supported lipid bilayer system, for example - one might use them to study protein-protein interactions at a membrane. Moreover, because SH light can be generated at non-planar surfaces (liposomes, for instance, where their diameter is  $\sim \lambda$ , the wavelength of the fundamental light; see Srivastava and Eisinger, 1998), they may also find use in studies involving the surface of liposomes or biological cells.

The present application has demonstrated the concept of a 'SHG-label': the labeling of some molecule of interest with a SH-active moiety for studying that molecule at an interface via a surface-selective technique such as second harmonic generation. As an illustration of the technique, the protein cytochrome c was covalently labeled with amine- and sulfhydryl-specific SH-active dyes in order to study the dye-protein conjugate at an air-water interface. Because of the SH-activity of the dye, the protein can be easily detected at the interface; if unlabeled, the protein is undetectable. Any protein of interest might thus be studied at an interface using a SHG-

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